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13. ABSTRACT *(Maximum 200 Words)*

A major goal of this Idea proposal is to determine whether the penetratin-based peptide delivery system may be developed to effectively deliver anti-cancer therapeutic peptides/proteins into breast cancer cells. We have fulfilled the objective 1 (Tasks 1-3) during the first year of the grant support. Although we experienced initial difficulties labeling the peptides with FITC, we finally were able to succeed in chemical synthesis of FITC-labeled penetratin peptides. When evaluating the translocation abilities of penetratin peptides in breast cancer cells, we were excited to find that the shortened FITC-YGRKKRRQR peptide was able to penetrate into breast cancer cells efficiently. Systematic studies of this penetratin delivery peptide in breast cancer cells, including the effective dose range, delivery efficiency, tolerable doses, stability etc. have led to better understanding of the properties of these peptides. Most importantly, we have found that the shortened FITC-YGRKKRRQR peptide can selectively translocate into the cytoplasmic subcellular compartment without going into nucleus as did the original penetratin peptide. These works pointed a very positive direction for the next grant-support year, when we will test the effect of erbB2 signal-blocking peptides using penetratin delivery system, which may be developed into new therapeutic agents.

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Introduction

It has been well recognized that the next frontier in molecular medicine is the delivery of therapeutics. Among the biological therapeutics, peptides/proteins are especially difficult and challenging to deliver. A peptide sequence localized within a 13-amino acid domain of Tat (named "penetratin"), when linked to other peptides or proteins, was able to carry attached peptide or protein into the cells when they were added to cell culture medium (1). We **hypothesized** that the unique property of penetratin can be utilized for delivery of therapeutic peptides/proteins. It should be noted that after the funding of this Idea Award, a report published in *Science* by Steven Dowdy's laboratory demonstrated that penetratin can deliver functional β-galactosidase protein (120 kD) to all tissues in mice (2). This report indicated that our basic hypothesis is right. However, although this report showed the high efficiency of the pennetratin system they used but the reported pennetratin system clearly has no selectivity or targeting ability that will limit its application for breast cancer treatment. The major goal of our Idea proposal is **to develop a new penetratin-based peptide delivery system that specifically targets** erbB2-overexpressing breast cancer cells. We have proposed three Specific Aims to fulfill our major goal. During the first funding year, we focused on Aim1, i.e. **to establish and optimize the penetratin delivery system for targeting.** We have fulfilled this Aim and identified a shortened penetratin sequence that deliver to the cytoplasmic subcellular compartment of cells but not the nucleus. Our effort has pointed a very positive direction for the next grant-support year, when we will test the effect of erbB2 signal-blocking peptides using penetratin delivery and start to develop penetratin-based delivery system targeting specifically erbB2-overexpressing breast cancer cells.

Body

During the first funding year, we have performed our studies focusing on Specific Aim 1.

Objective 1 (Aim 1). To establish and optimize the penetratin delivery system.

Task 1: Chemical synthesis of biotin-labeled penetratin peptides (P1, P2, P3, P4) using a solid phase automated peptide synthesizer.

Initially we tried to synthesize the following panel of Tat penetratin peptides by a solid phase automated peptide synthesizer.

peptide P1	biotin-YGRKKRRQRRRPPQC
peptide P2	biotin-YGRKKRRQRRRC
peptide P3	biotin-YGRKKRRQRRC
peptide P4	biotin-YGKKKKKKKKKC

To save time and budget, we first tested if P2 can deliver as effectively as P1. Indeed, P2 without the PPQ sequence was shown to deliver as effective as P1. After that, the publication of the *Science* paper by Steven Dowdy's laboratory promoted us to change the peptide labeling strategy.

(1) Since they have success in using FITC fluorescence labeling, we decide to change from biotin labeling to FITC labeling for future experimentation. The advantage of this change is that

FITC emits fluorescence that can be visualized without fluorescein-labeled secondary detection whereas biotin labeling does need detection by fluorescein-linked streptavidin which binds biotin. (2) Since the linear peptide is stable enough for delivery, we deleted the C-terminal cysteine residual. (3) Since our major goal is to develop penetratin peptide that can target breast cancer cells, we decided to test if we can reduce the defuse penetration of the old version penetratin to more specific subcellular compartment by further delete RR (new P3) from the old version penetratin. Thus, we set out to synthesize the following peptides:

peptide P1	FITC-YGRKKRRQRRR-OH
peptide P2	FITC-YGRKKRRQRR-OH
peptide P3	FITC-YGRKKRRQR-OH
peptide P4	FITC-YGKKKKKKKK-OH

However, we were unable to directly link FITC to our peptides. After two cycle failures, we realized that we need to add an β-Alanine bridge between FITC and our desired peptides. Thus, the peptides we were able to synthesize are the following:

peptide P1	FITC-β-Ala-YGRKKRRQRRR-OH
peptide P2	FITC-β-Ala-YGRKKRRQRR-OH
peptide P3	FITC-β-Ala-YGRKKRRQR-OH
peptide P4	FITC-β-Ala-YGKKKKKKKK-OH

Task 2: Evaluation of the translocation abilities of the above penetratin peptides in breast cancer cells. We have treated the SKBR3 and MCF-7 human breast cancer cell lines with the above peptides (Fig. 1). Penetratin-treated cells were fixed and peptide internalization were visualized under fluorescence microscope. Our data showed that all three peptides (P1, P2, P3) can translocate inside cells. Importantly, the P3 peptide is predominantly localized in the cytoplasmic compartment of the cells, whereas the P1 peptide is diffusely distributed all over the cells. This result suggest that the P3 peptide would be an excellent delivery vehicle for delivering the blocking peptides of ErbB2 signaling in the cytoplasmic compartment of the cells without nuclear toxicity.

Task 3: Systematic study of penetratin delivery system.

MCF-7 breast cancer cells were cultured in P3 penetratin-containing medium and the biological properties of penetratin in culture were determined (see Table below).

Penentratin Concentrations	Fluorescence Intensity	Background	Cell Shape Changes
30 μM	+++	+	+
3 μM	+	+/-	-
300 nM	+/- (longer time +)	-	-
30 nM	-	-	-

The above preliminary results indicate that the effective dose range of penetratin is quite broad from 300 nM and up. The delivery efficiency is sufficient without inducing significant cytotoxicities. The tolerable dose is under 30 μM. The peptides seem to be stable during the experimentation procedures. For effective and specific delivery without significant background and toxicity, a final penetratin concentration of 1 – 3 μM is recommended.

Note: Since we lost some time dealing with unexpected problem on FITC labeling of penetratin, we will need to work very hard to catch the lost time and may also need to modify our experimental plans accordingly.

Key Research Accomplishments

Although we experienced unexpected problem, we have fulfilled the Specific Aim 1 during the first year of the grant support.

1. We have chemically synthesized the FITC-labeled penetratin peptides (P1, P2, P3, P4).

2. We demonstrated for the first time that the (FITC-β-Ala-YGRKKRRQR-OH peptide can effectively translocate into breast cancer cells and specifically localize in the cytoplasmic compartment.

3. We have investigated the effective dose range of penetratin, the delivery efficiency, the tolerable dose and the stability of peptides.

Reportable Outcomes:

-manuscripts, abstracts, presentations

We are not to the stage of writing manuscript yet.

-patents and licenses applied for and/or issued

We intend to patent our findings in the future.

-degrees obtained that are supported by this award	None
-development of cell lines, tissue or serum repositories	None
-informatics such as databases and animal models	None
-funding applied for based on work supported by this	None
-employment or research opportunities applied for and/or received on experiences/training supported by this award	A post-doctoral fellow

Conclusions:

The research supported by the first year of funding has been satisfactory. Although we had unexpected problems with FITC labeling of the peptides, we have fulfilled the major goal for the first year of the grant support. We succeeded in chemical synthesis of FITC-labeled penetratin peptides. We were excited to find that the shortened FITC-YGRKKRRQR peptide was able to penetrate into breast cancer cells efficiently. Systematic studies of this penetratin delivery peptide in breast cancer cells have led to better understanding of the properties of these peptides. Most importantly, we have found that the shortened FITC-YGRKKRRQR peptide can selectively translocate into the cytoplasmic subcellular compartment without going into nucleus as did the original penetratin peptide. These works pointed a very positive direction for the next grant-support year, when we will test the effect of erbB2 signal-blocking peptides using penetratin delivery system.

References:

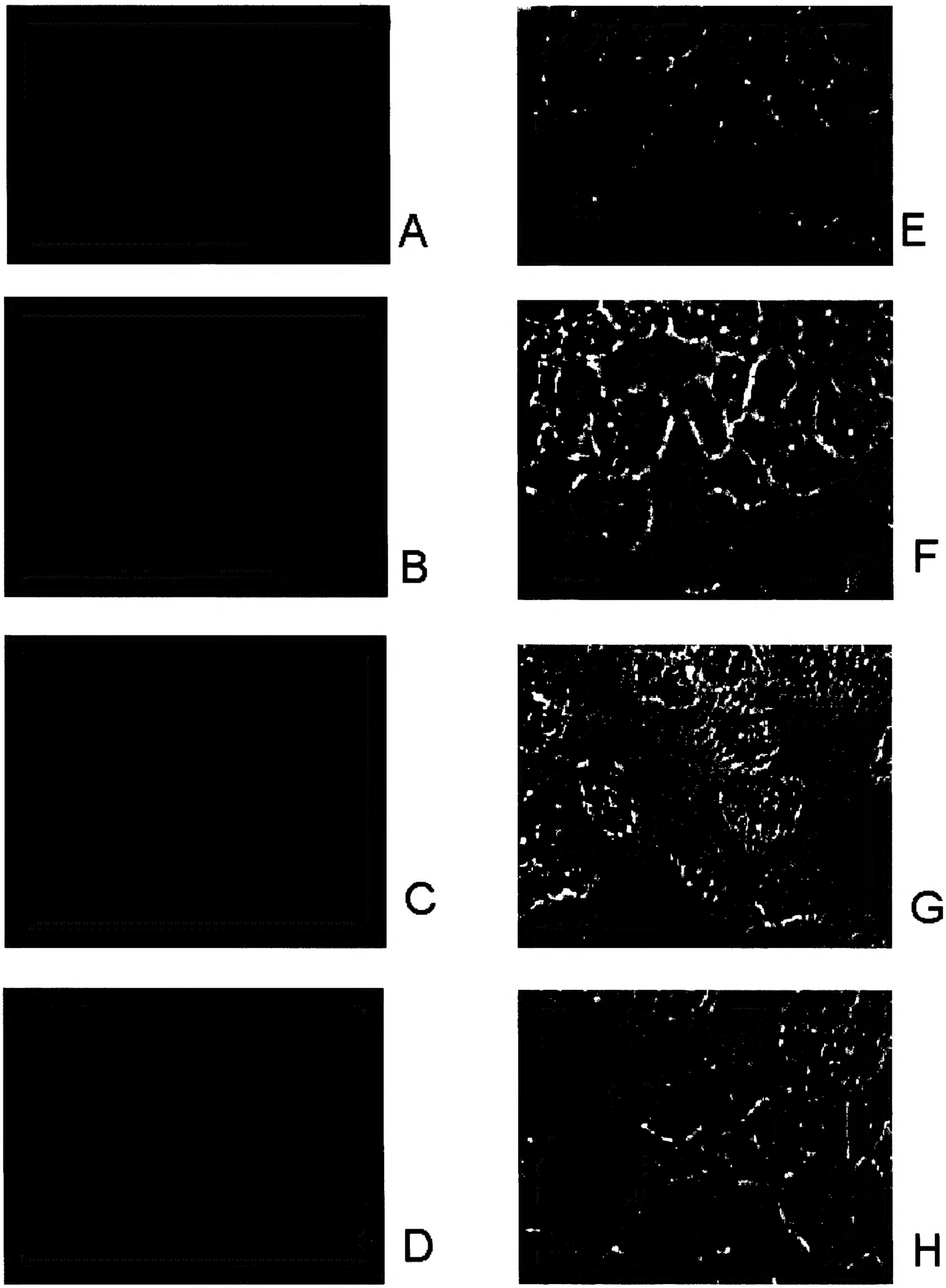
1. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. Tat-mediated delivery of heterologous proteins into cells, Proc. Natl. Acad. Sci. USA. *91:* 664-668, 1994.

2. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. In vivo protein transduction: delivery of a biologically active protein into the mouse, 1999.

Appendix

One figure cited in this report (see next page).

Figure 1



Legend: Subcellular distributions of penetratin peptides in SKBR3 cells

SKBR3 cells grown on sterile coverslips were incubated with a panel of peptides respectively at 10μM for 15 min. Cells were then fixed with 4% paraformaldehyde in PBS and washed with PBS 20 times.

Panel A-D show fluorescent images of the cells treated with Peptide1 (FITC-β-Ala-YGRKKRRQRRR, positive control), Peptide2 (FITC-β-Ala-YGRKKRRQRR), Peptide3 (FITC-β-Ala- YGRKKRRQR), and Peptide4 (FITC-YGRKKKKKKKKK, negative control), respectively. Panel E-F are light field images corresponding to Panel A-F, respectively.

Panels A and B show Peptide1 and 2 distribute in nucleus as well as cytoplasmic region. In contrast, Peptide 3 are observed predominantly in cytosol in panel C. In negative control cells (panel D), FITC signaling are only detected outside of the cells. These cells were examined and photographed by Nikon ECLIPSE E400 and the images were processed by MetaMorph Imaging System (Universal Imaging Corporation, PA) All photographs were taken at the same magnification (x400).